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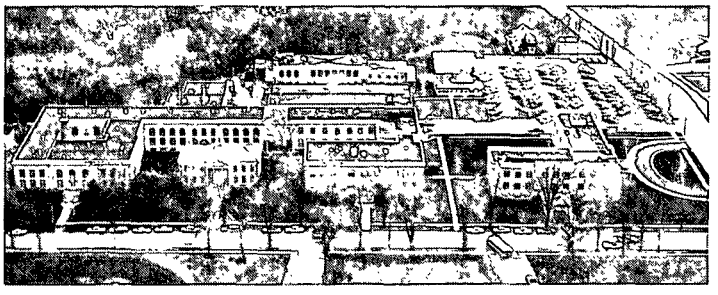

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SOME PROPERTIES OF A XYLOGLUCAN ISOLATED FROM THE SPENT MEDIUM OF SUSPENSION CULTURED LOBLOLLY PINE (*PINUS TAEDA*) CELLS

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Portions of this work were used by LTN as partial fulfillment of the requirements for the Ph.D. degree at The Institute of Paper Chemistry. This manuscript has been submitted for consideration for publication in Plant Physiology

Xyloglucan from Loblolly Pine

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Some Properties of a Xyloglucan Isolated from the Spent Medium of Suspension Cultured Loblolly Pine (*Pinus taeda*) Cells

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[2]Abbreviations: PEPS, pine extracellular polysaccharides; DEAE PEPS, PEPS from which arabinogalactan was removed by DEAE Sephadex chromatography; Mw, weight average molecular weight; Mn, number average molecular weight.

ABSTRACT

A xyloglucan isolated from the spent medium of suspension cultured loblolly pine (Pinus taeda) cells was easily separated from an arabinogalactan but was strongly bonded to protein with two types of bonding characteristics. The protein was completely removed by a sequential treatment with a protease and deesterification. Acid hydrolysis yielded 44.3% glucose, 30.4% xylose, 8.1% galactose, 2.4% arabinose and 4.8% fucose. Partial characterization of this polysaccharide by methylation showed it was a 1,4-glucan (probably β-linked) branched at the 6 position with short chains of xylose, galactose, arabinose and fucose. Fragmentation with a fungal cellulase yielded a number of glucan fragments, one of which was xylose rich and others in which galactose was the predominant branch constituent. These fragments did not display any biological activity. The pine xyloglucan was polydisperse in water and had Mw and Mn of 117,500 and 38,000, respectively. A xylose-containing oligosaccharide devoid of uronic acid was also isolated from the spent medium.

Xyloglucans found in the primary cell wall of many plant cells and in the growth medium used to propagate these cells have been the subject of much research in the last twenty years. Although composed primarily of xylose and glucose, varying amounts of other hexoses, deoxy hexoses and pentoses are also present. These xyloglucans have been shown to be involved with cell elongation (12,22,26). They have been isolated from a number of different sources including sycamore (3), dwarf french beans (16) and apples (2,18).

Few investigations of coniferous sources have been reported. Earlier research from this laboratory (24) detected a xyloglucanlike polysaccharide in the cambial cells of jack pine but not in recently matured tracheids. It was not determined whether it was a transient structural component or a reserve carbohydrate (amyloid). Similar polymers have been isolated and characterized from the cambial tissues of aspen, basswood and sugar maple as well as balsam fir compression wood holocellulose by Timell and coworkers (19,20).

Other investigators have looked at the polysaccharides in developing conifer tissues (15,25) and have reported xyloglucans in Douglas-fir (23) and radiata pine (13) tissues. The most recent and extensive work to date describes all of the extracellular polysaccharides of suspension-cultured sycamore cells. Overall, investigation of coniferous xyloglucans has been overshadowed by the enormous amount of work on the xyloglucans of dicotyledons (21).

In this paper, a tentative structure for a xyloglucan isolated from the spent medium used to culture loblolly pine cells is proposed along with information on its molecular size, associated polymers in suspension medium, and a brief view of cellulase-produced xyloglucan fragments. The pine xyloglucan is also compared to other isolated xyloglucans, especially that from Douglas-fir (23) and the xyloglucan isolated from the suspension culture medium of sycamore cells (21).

MATERIALS AND METHODS

Growing the Cells and Harvesting Spent Medium. Loblolly pine (Pinus taeda) cells [cell line 2F, a pooled source initiated from immature embryos on LM medium with 2,4-D (14)] were suspension cultured in LM 3 medium [LM 3 differs from LM as follows (in mg/L): 370 rather than 1850 $MgSO_4 \cdot 7H_2O$; 1538 $MgNO_3 \cdot 6H_2O$ (not present in original LM); 250 rather than 100 myo-inositol]. Cultures were maintained in darkness on reciprocating shakers (46 rpm). The cells were subcultured every 12 to 14 days after which the spent medium was collected by filtration on coarse sintered glass.

Isolation of Pine Xyloglucan. As much spent medium as could be handled was immediately added to 4 volumes of 95% ethanol. Any remaining spent medium was frozen for future use. The resulting precipitate was collected by centrifugation, redissolved in water, and reprecipitated in 4 volumes of 95% ethanol. These solids were collected by centrifugation, dissolved in water, and freeze dried. This fraction was called pine extracellular polysaccharides (PEPS).[2] The yield of PEPS from the stored, frozen medium was always less than that from the medium worked up immediately.

The PEPS (up to 300 mg) was dissolved in 10 mM potassium phosphate buffer (1 ml), pH 6.5, and placed on a DEAE Sephadex, A 25-120 (Sigma Chemical) column (9 x 150 mm). The column was eluted with the same buffer and the fractions monitored for xyloglucan with an iodine assay (11). The xyloglucan rich fractions were pooled, desalted with a mixed bed resin (M 614, Baker Chemical), and freeze dried. This fraction was treated with the protease, Pronase (Calbiochem), (1:5, Pronase:polysaccharide, wt:wt) for 4 to 5 hours at 32°C. The resulting solution was separated on a similar column (9 x 150 mm) of DEAE Sephadex and the

xyloglucan rich fractions pooled and freeze dried. This preparation was deesterified by the method of Bauer et al. (3).

Isoelectric Focusing. Isoelectric focusing was performed on a LKB 2117 Multiphor, with a flat bed of polyacrylamide gel. Samples (20 µl) of a 5 mg/ml solution of crude polysaccharides were placed on the gel. One half was stained for carbohydrates (10), the other half for proteins with Coomassie blue (5).

General Analysis. The TAPPI (4) method was used for total carbohydrate analysis. Carbon, hydrogen, oxygen and nitrogen analysis was performed by Micro Tech Laboratories, Inc. of Skokie, Ill.

Preparation for GC and GC/MS Analysis. The xyloglucan or xyloglucan fragments were methylated using techniques described by Cuicanu and Kerek (6). The methylated polysaccharide was hydrolyzed, reduced and acetylated following the procedures described by Albersheim et al. (1). Prereduction with $NaBD_4$ was performed in the same manner as $NaBH_4$ reduction.

GC/MS and GC Analysis. The GC/MS analyses were performed on a Hewlett Packard 5985 GC/MS instrument with a 2 mm x 6 ft glass column packed with SP 2340 (Supelco Inc.). Runs were made with a temperature program to hold at 170°C for 13 min and then increase at 3°C/min to 220°C. The GC analyses were done on a Packard Model 417 with a SP 2340 packing in a 2 mm x 6 ft glass column and run isothermally at various temperatures ranging from 150 to 190°C.

Authentic samples of many partially methylated sugars (9) were used as GC and MS controls and for calibration of columnns by comparison with published data.

Enzymatic Degradation. Deesterified pine xyloglucan (200 mg) was dissolved in 20 ml of 50 mM sodium acetate buffer (pH 5.0). Cellulase (100 units) (Cellulysin, Calbiochem, from Trichoderma viride) and 0.01% (w/w) sodium azide

were added and the solution shaken for 48 hours at 40°C. One unit of enzyme liberated 1 μmole of glucose from cellulose in 1 h at pH 5.0 and 37°C.

Gel Filtration of Degraded Material. The above cellulase treated material was desalted on Dowex AG50W X8 (H+) resin, evaporated to a few ml, filtered, and placed on a BioGel P2 (200 to 400 mesh) (Bio Rad) column. The column was 1 x 90 cm and was kept at 55°C. In the initial separation, 3 ml fractions were collected at a flow rate of 5 ml/h. The pooled fractions comprising the main peaks of this collection were placed on the column again and 1 ml fractions collected. One hundred microliter samples of the fractions were assayed for total carbohydrate using a phenol sulfuric acid test (7).

Isolation of Nonprecipitable Polysaccharides of Culture Medium. The ethanol-medium mixture remaining after the PEPS had been collected was let stand in a hood until most of the ethanol had evaporated. The remaining liquid was evaporated to a syrup. This syrup was placed on chromatographic paper and eluted with a 6:3:4 (v:v:v) mixture of ethyl acetate:acetic acid:water. Migration rates in this and other solvents approximated that of xylopentaose. Chromatograms were stained with silver nitrate or *p*-anisidine to detect organic material and reducing groups. Hydrolyzed xylans were analyzed by paper chromatography using an 18:3:1:4 (v:v) ethyl acetate, acetic acid, formic acid, water solution.

Determination of Molecular Size. A column (1 x 120 cm) of Sepharose CL 6B (Pharmacia Fine Chemicals AB) was calibrated with four dextrans of known size and a blue dextran to determine void volume. All of these dextrans were purchased from Pharmacia Fine Chemicals AB. A small sample (5 mg) of pine xyloglucan was dissolved in 0.5 ml of 0.1 N NaOH and eluted with 0.1 N NaOH at a rate of 8 ml/h. Fractions (1 ml) were collected and analyzed with the phenol sulfuric acid assay (7).

Pea Stem Elongation. The procedure described by York et al. (26) was followed except only one segment was excised from each stem.

RESULTS AND DISCUSSION

Isolation of the Pine Xyloglucan. The spent suspension medium from the proliferative growth of pine cells yielded approximately 500 mg solids from each liter of suspension precipitated with ethanol. Cultures which grew poorly were abandoned, since they exhibited a diminished yield of xyloglucan and resulted in cells rich in starch. Suspected pectinaceous material that did not redissolve in water comprised 70% of the precipitable solids from the medium. The 30% of the material that redissolved in water was called pine extracellular polysaccharides (PEPS) and its composition is given in Table I.

The next isolation stage (Fig. 1) was similar to that used by Bauer et al. (3) to isolate xyloglucan from sycamore spent medium. An arabinose, galactose rich polysaccharide was removed from the xyloglucan portion of the PEPS by DEAE Sephadex chromatography. The carbohydrate composition (DEAE PEPS in Table I) resembled reported xyloglucans (3,16) but contained a large amount of protein. Isoelectric focusing showed the protein was strongly (covalently) attached to the xyloglucan. A disproportionate amount of protein remained with this neutral fraction, indicating that in the medium, proteinaceous material as well as the arabinogalactan was associated with the neutral xyloglucan. Most of the protein was removed from the xyloglucan with a protease to leave a fraction called protein-free DEAE PEPS. This fraction still contained some protein and was 75% carbohydrate.

Finally the sample was deesterified. It had been hoped that the xyloglucan could be isolated without this step because alkali-labile groups have been

reported in xyloglucans. Deesterification removed the remaining protein and left the xyloglucan preparation over 90% carbohydrate. Analysis for carbon, hydrogen and oxygen was consistent for carbohydrate and suggested the remaining 10% to be traces of unidentified carbohydrates and some inorganic material.

Some other isolation techniques were attempted. Treating the crude PEPS with the protease before anion exchange chromatography produced a fraction that still contained 3.9% nitrogen, as compared to 1.3% in the protein-free DEAE PEPS. Deesterification of PEPS before anion exchange chromatography resulted in a fraction comprised of 62% carbohydrate and 2.9% nitrogen.

These techniques not only produced a reasonably pure xyloglucan but shed light on its association with other materials in the medium. Xyloglucan, unlike the xylan, behaved as a large molecule to which considerable amounts of protein are attached. The complexity of this extracellular material suggests it might not be an exudate but perhaps originates instead from the remnants of cell walls that have sloughed off during cell growth and division and through agitation. If true, the structure of the xyloglucan from the spent medium could elucidate some of the features of the primary cell wall.

The arabinogalactan separated from the original mixture by DEAE Sephadex was only loosely associated with the xyloglucan protein complex. It may have simply coprecipitated with the xyloglucan fraction. The arabinogalactan fraction also contained far less protein as can be inferred from Table I. Deesterification of the PEPS prior to anion exchange chromatography resulted in more arabinogalactan being present in the neutral fraction.

The different purification procedures yielded information on the xyloglucan protein complex. There appeared to be two types of protein bonded to the xyloglucan. One portion of the protein was removed by a protease. The failure to remove all of the protein could have been due to steric factors. The second

type of protein was one that was removable only by deesterifying the polysaccharide and the breaking of ester bonds. Crude PEPS was also treated with the protease before anion exchange. Less protein was removed from the xyloglucan suggesting the presence of the arabinogalactan interfered with the action of the enzyme. The existence of two types of protein was reinforced by the fact that deesterification prior to anion exchange failed to remove all of the protein.

Methylation of the Xyloglucan. The general structure of the pine xyloglucan was investigated through derivatization and hydrolysis of the polysaccharide to its methylated alditol acetates and subsequent GC and GC/MS analysis. Only the methylation technique differed from methods used by others. The method of Ciucanu and Kerek (6) was found to methylate quickly and completely while being far less tedious to perform than variations of the Hakamori method (8).

The various permethylated alditol acetates were identified by their GC retention times and mass spectra. These retention times appear in Table II and the total ion chromatogram of the deesterified xyloglucan can be seen in Figure 2. The pine xyloglucan configuration shown in Figure 3 resembled xyloglucans isolated from other sources such as cultured sycamore cells (3), runner beans (16), and many others (2,15,25). It appeared to have more unbranched glucose units than most of the other reported xyloglucans. When compared to the extracellular xyloglucan from sycamore spent medium (3), the outstanding feature of this xyloglucan is the greater amount of terminal galactose units. The recently reported xyloglucan from Douglas-fir (23) also contains relatively large amounts of terminal galactose units, and this may be a feature common to coniferous xyloglucans.

Molecular Size. A sample of protein-free deesterified pine xyloglucan was run on a calibrated column of Sepharose to determine its molecular size. The

elution pattern of the xyloglucan was monitored using the phenol sulfuric acid assay on 1 ml fractions. A graph of absorbance vs. fraction number can be seen in Figure 4. One broad peak with a slight shoulder was found which control experiments suggest is real and not due to experimental factors. The apex of the peak corresponds to a dextran molecular weight of 270,000 Daltons or a xyloglucan containing more than 300 sugar units. The calculated weight (Mw) and number (Mn) average molecular weights were 117,500 and 38,000, respectively. The pine xyloglucan from the suspension medium was fairly large (with respect to other reported xyloglucan molecular weights (15,26) and polydisperse. The slight shoulder may result from the crosslinking action of the residual protein in a manner similar to that observed for basswood arabinogalactan complexes (20).

Gel Permeation Chromatography of Cellulase Degraded Xyloglucan. De-esterified pine xyloglucan was digested with a commercial cellulase (Cellulysin, CalBiochem) derived from T. viride. The digestate was eluted on a BioGel P2 column that had been calibrated with maltoheptose, raffinose, stachyose, cellobiose and glucose. Sycamore xyloglucan (3) has been reported to yield two major peaks under these conditions, nonasaccharide, and a heptasaccharide. The pine xyloglucan fragments also have two peaks in their elution pattern (Fig. 5) that coincide with fragments of this size. The "C" and "D" peaks of the xyloglucan fragments are most probably a nona- and a heptasaccharide, respectively. The remaining peaks comprise a significant portion of the enzymatically cleaved xyloglucan. Peaks D and G are made up of fragments ranging from 5 or 6 sugar units to monosaccharides. The pine xyloglucan produced far more of these smaller fragments than the sycamore xyloglucan (3) and more closely resembled the xyloglucan from runner beans (17).

If the cellulase consumes all of the unbranched glucose units except those that are at the reducing end of branched fragments, then the unbranched glucan portion of the xyloglucan occurs in short sections between small branched portions of the glucan backbone. The structure of the pine xyloglucan would then resemble the proposed runner bean xyloglucan more closely than some of the large blocks of branched sections proposed by Bauer (3).

The fractions comprising each peak eluted from the BioGel P2 column were placed on the column and eluted again. Appropriate fractions were pooled and modified for GC and GC/MS analysis. The procedure was the same as used for the xyloglucan polymer except that the fragments were prereduced with sodium borodeuteride before methylation. To this end, a cellobiose sample was prereduced and derivatized to produce a standard of 1,2,3,5,6-penta-O-methyl-4-O-acetyl glucitol. This standard was chosen because of the likelihood of unbranched glucose units being at the reducing terminus of the cellulase cleaved xyloglucan fragments. This was what had been reported earlier with sycamore xyloglucan (3). A sample of tamarind xyloglucan was fragmented by the aforementioned technique and one fragment analyzed by GC. This tamarind fragment had a reducing terminus of unbranched glucose which was easily seen in the gas chromatogram. Due to lack of material, the "C" subfractions (4 of them) were later pooled and run again on the GC. The "E" subfractions were also pooled and rerun. These fragments, however, still did not yield significant specific structural information.

Generally, xyloglucans are thought to be blocks of branched sections separated by unbranched glucan backbone. The size of these unbranched sections was unknown, but large unbranched sections were not likely to be present due to the small size of the fragments produced by cellulase cleavage. There was only

enough unbranched glucose present in the polymer to separate branched sections with one or two glucose units. The composition of the branched blocks then revealed some of the "finer" structure of the xyloglucan. One of the "B" subfragments had only one identifiable major peak. It was the acetate of 2,3,6-tri-O-methyl glucitol, indicating an unbranched glucose unit at the nonreducing end of the fragment. The pooled "C" peak provided more information. The major resolved components can be seen in Figure 6. This octa- or nonasaccharide contains some unbranched and some branched glucose units at the reducing end, indicating a mixture of fragments in the sample. This fraction also contained terminal galactose and glucose units, the former being one of the predominant peaks. This fragment did not contain any xylose or fucose, even though it was of a size similar to fucose-containing xyloglucan fragments observed by others. The chromatogram of one of the "D" subfragments contained several large identifiable peaks. The methylated alditol acetates present in substantial amounts were acetates of 2,3,4-O-methyl xylose, 1,2,3,5 tetra-O-methyl glucitol, and 3,4,6-tri-O-methyl galactose. Lesser amounts of the 2,3,4,6-tetra-O-methyl galactose, 2,3-di-O-methyl xylose, and 2,3-di-O-methyl glucose acetates were found. This was the only fragment that had a substantial amount of xylose. Although no fucose was evident in the chromatogram either, the composition of this fragment resembled other fucose-containing xyloglucan fragments (1,26). There was, however, no reason to expect the loss of fucose. The pooled "E" fractions also provided enough data to ascertain the general structure of this oligosaccharide. This hexa- or pentasaccharide contained a large amount of 2,3,4,6-tetra-O-methyl galactose acetate. These terminal units were presumed in other xyloglucans to be mostly bonded to the glucan backbone via a xylose unit. This fragment may have galactose units bonded directly to the backbone.

The information from these fragments, although not complete, showed the pine xyloglucan to be different than other isolated xyloglucans. The xylose seemed to be contained in one set of fragments, while terminal galactose units were bunched in other pieces.

Xylan Oligosaccharide. A small xylan oligosaccharide was isolated from the ethanol solution remaining after precipitation of the extracellular polysaccharides. Large quantities of this ethanol/media solution were evaporated to a syrup. Paper chromatography was used to separate the xylan from the sucrose, which was the sugar source in the media. Paper chromatography also showed this xylan to be 5 xylose units in size and to contain no uronic acids. Xylans are a major hemicellulose component of secondary walls and always contain uronic acids (9) but are not often found in primary wall tissue and have not been isolated from suspension culture media. This xylan oligosaccharide was derivatized for GC and the chromatogram obtained. The only identifiable peak was that of a di-O-methyl xylose (either 2,3 or 3,4), indicating a 1,2 or 1,4 linked unbranched xylan.

Pea Stem Elongation. The mixture of the fragments of cellulase digested pine xyloglucan (elution Figure 4) was tested for inhibitory activity in auxin-stimulated elongating pea stems. The procedures of York et al. (26) were followed with treatments ranging in concentration from 10 to 0.001 µg/ml of xyloglucan fragments in the growth medium. Controls without xyloglucan fragments plus and minus 2,4-D elongated consistently. However, over eight trials with 12 stem segments in each treatment, no repeatable statistically significant inhibition was seen for any concentration of xyloglucan fragments. Since none of the larger xyloglucan fragments were shown to contain fucose, a component of the reported active xyloglucan nonasaccharide (1), it is not surprising that the xyloglucan fragments did not affect the elongating pea stems.

CONCLUSIONS

The spent medium of suspension grown loblolly pine cells contained an arabinogalactan, a xylose-containing oligosaccharide, probable pectic substances and a xyloglucan-protein complex. Since the protein component could only be removed by Pronase treatment followed by deesterification, it was likely covalent bonds were involved. This separation could not be achieved if the arabinogalactan were not first removed nor if a reverse deesterification and Pronase sequence were employed. It is speculated that hindrance of the enzyme action and different proteinaceous linkages are involved.

Methylation data for the xyloglucan showed the presence of a (1,4)-linked glucan backbone, sections of which were unbranched. Other portions of the backbone contained branches from the 6 position of the glucose units, composed of xylose, galactose, arabinose, and fucose units. The composition of the pine xyloglucan resembled other reported xyloglucans, including one isolated from the spent medium of suspension cultured sycamore cells (21). It differed by having a relative abundance of terminal galactose units. This feature is characteristic of xyloglucan from the primary cell walls of Douglas-fir as well (23). The molecular size of the pine xyloglucan was determined to be 70,000 Daltons from which Mw and Mn values were calculated to be 117,500 and 38,000, respectively.

Cellulase digestion of the pine xyloglucan produced fragments of a wide size range. One of these fragments was rich in xylose, while others were galactose rich. This suggests that the branched sections of the xyloglucan were specific in composition. Xylose-containing branches were bunched together and separate from other branch types.

The cellulase-produced fragments showed no inhibitory effect on auxin stimulated pea stem elongation.

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Table I. Composition of Isolated Neutral Fractions

Component	PEPS	DEAE-PEPS	Protease-Treated	Deesterified	Initially Deesterified
		Mole% of Carbohydrate Fraction			
Fucose	1.8	3.5	4.8	4.8	3.6
Arabinose	11.7	1.8	2.5	2.4	8.5
Xylose	5.7	15.9	21.2	30.4	13.6
Mannose	1.2	1.1	1.4	0.9	1.4
Galactose	22.1	5.1	8.6	8.1	15.1
Glucose	8.4	23.6	35.9	44.3	19.5
Total Carbohydrate (wt.%)	52.1	51.0	74.4	90.9	61.7
Nitrogen (wt.%)	3.8	4.7	1.3	< 0.3	2.9

Table II. DEXG-3 Methylation Data Taken From GC of TFA Hydrolyzed, Alditol Acetates of Methylated Xyloglucan Run Isothermally at 170°C on SP-2340 and MS Analysis

RT, min	Area, %	Resolved Species
3.63	1.94	Unknown
6.10	2.12	235Me Ara
7.33	2.02	234Me Fuc
7.98	20.40	234Me Xyl
10.43	1.77	Unknown
14.64	6.20	2346Me Gal
18.79	6.46	23Me Xyl
29.43	4.66	346Me Gal
32.62	19.62	236Me Glc
68.53	32.36	23Me Glc

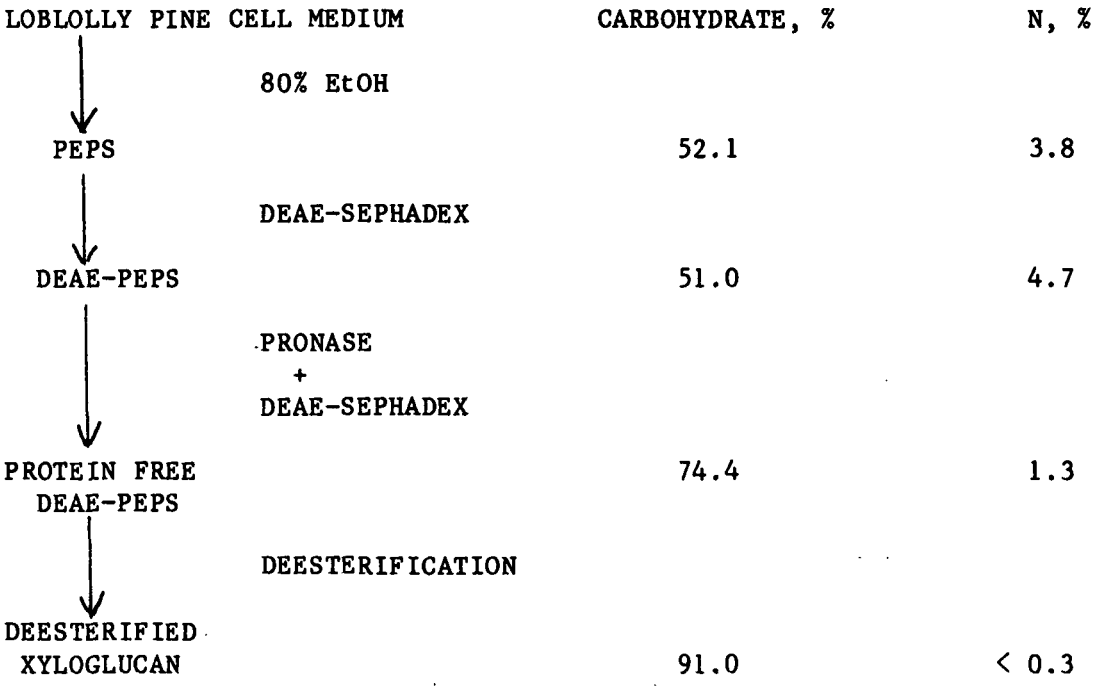

FIG. 1. Pine xyloglucan isolation procedures.

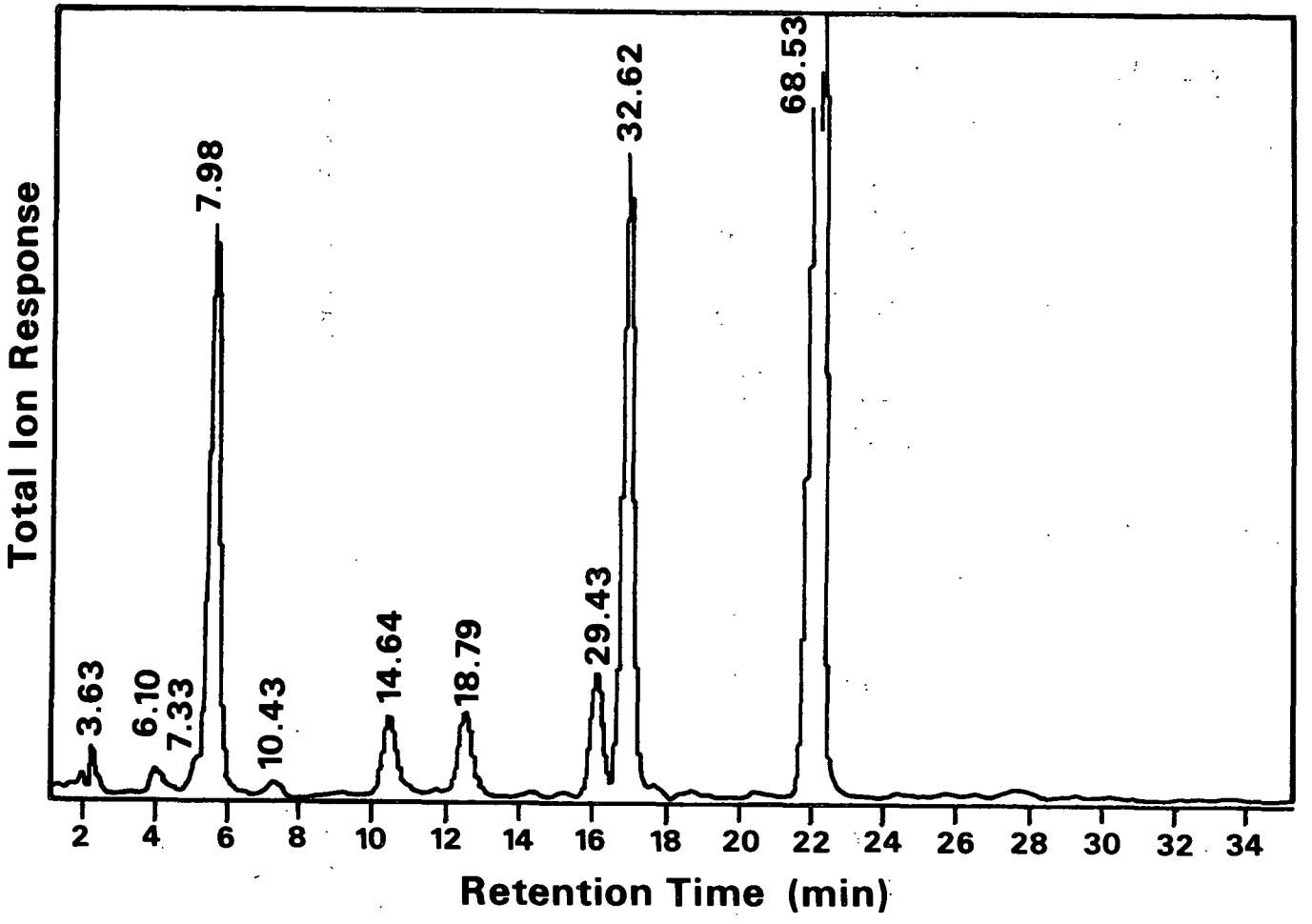

FIG. 2. Total ion chromatogram of DEXG-3. Partially methylated alditol acetates run on SP-2340, 150-220°C at 2°/min. Peaks labeled with retention times from isothermal gas chromatography on SP-2340 at 170°C.

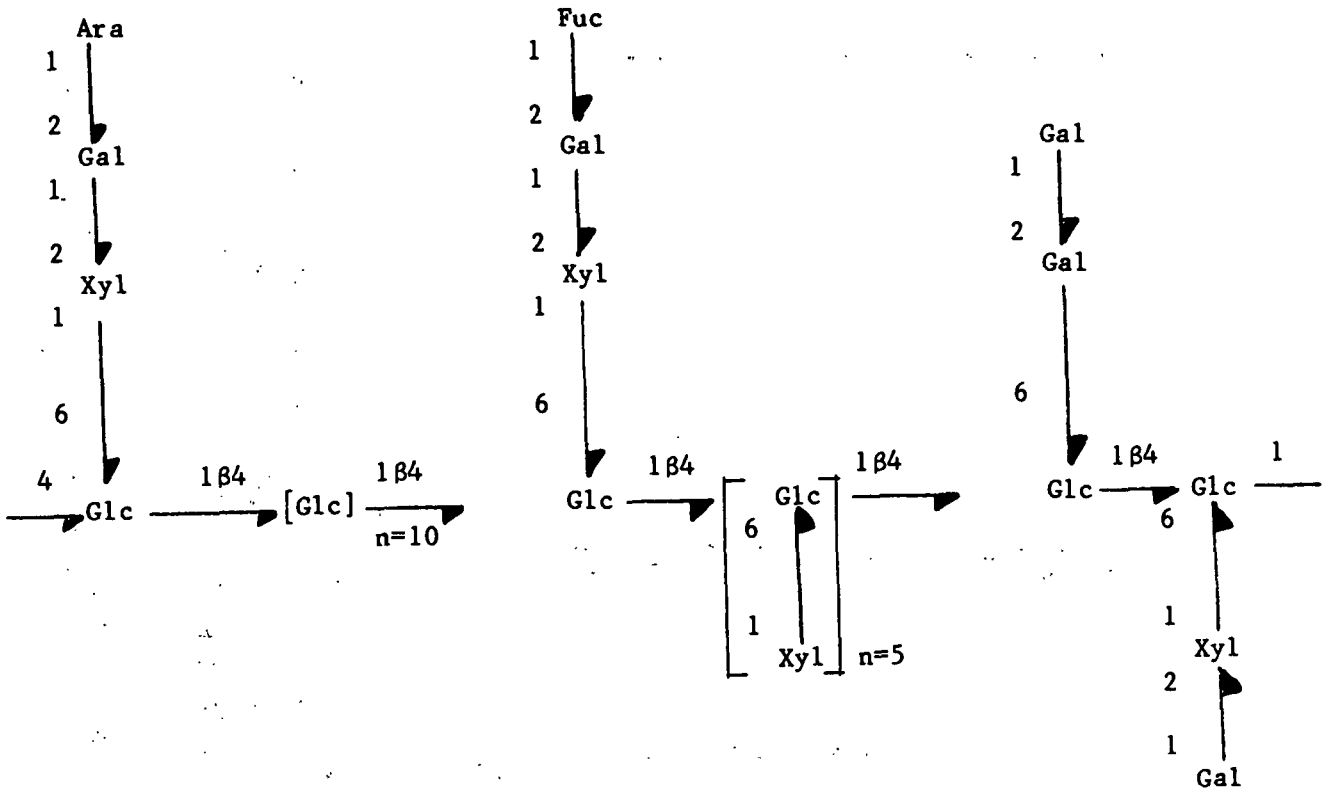

FIG. 3. Proposed pine xyloglucan structure. Branches have been placed arbitrarily on the glucan backbone.

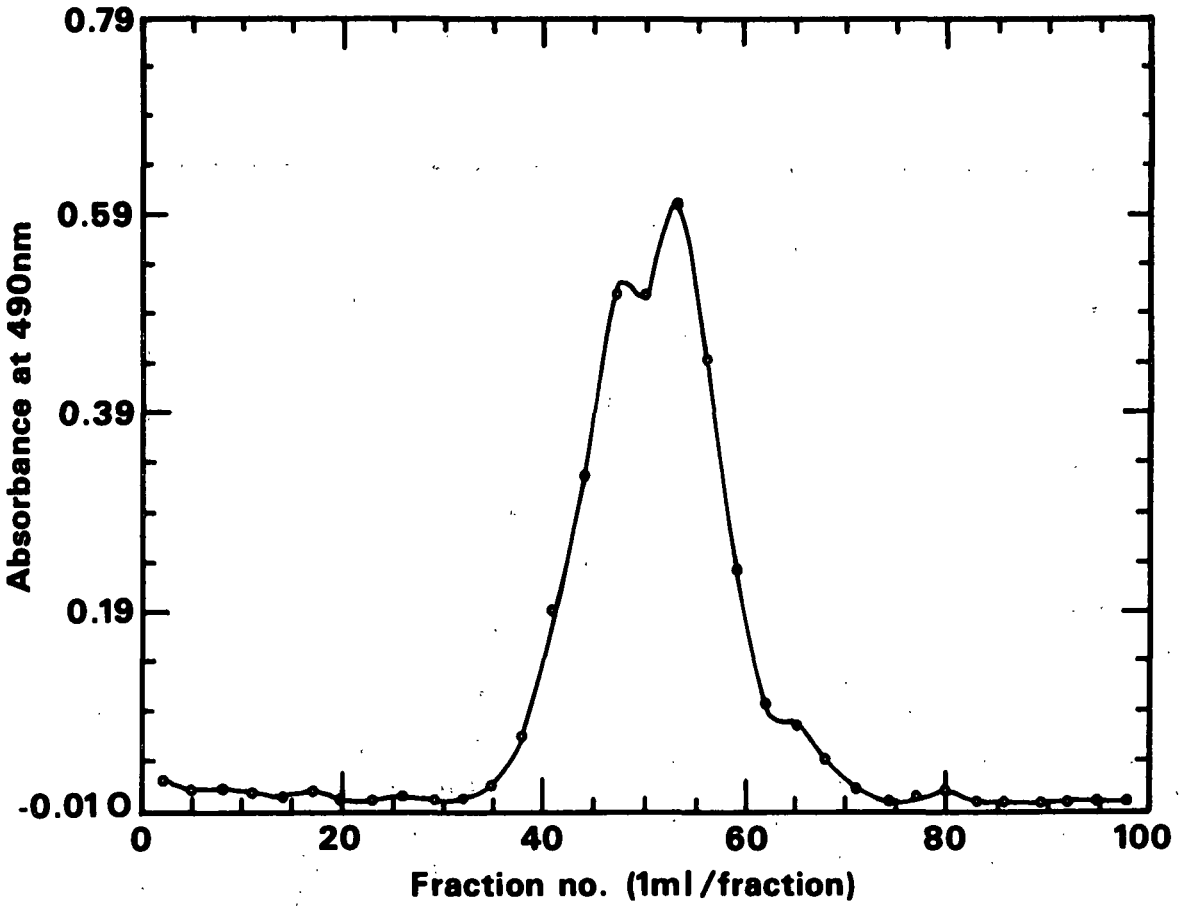

FIG. 4. Elution pattern of DEXG-5 on Sepharose CL-6B.

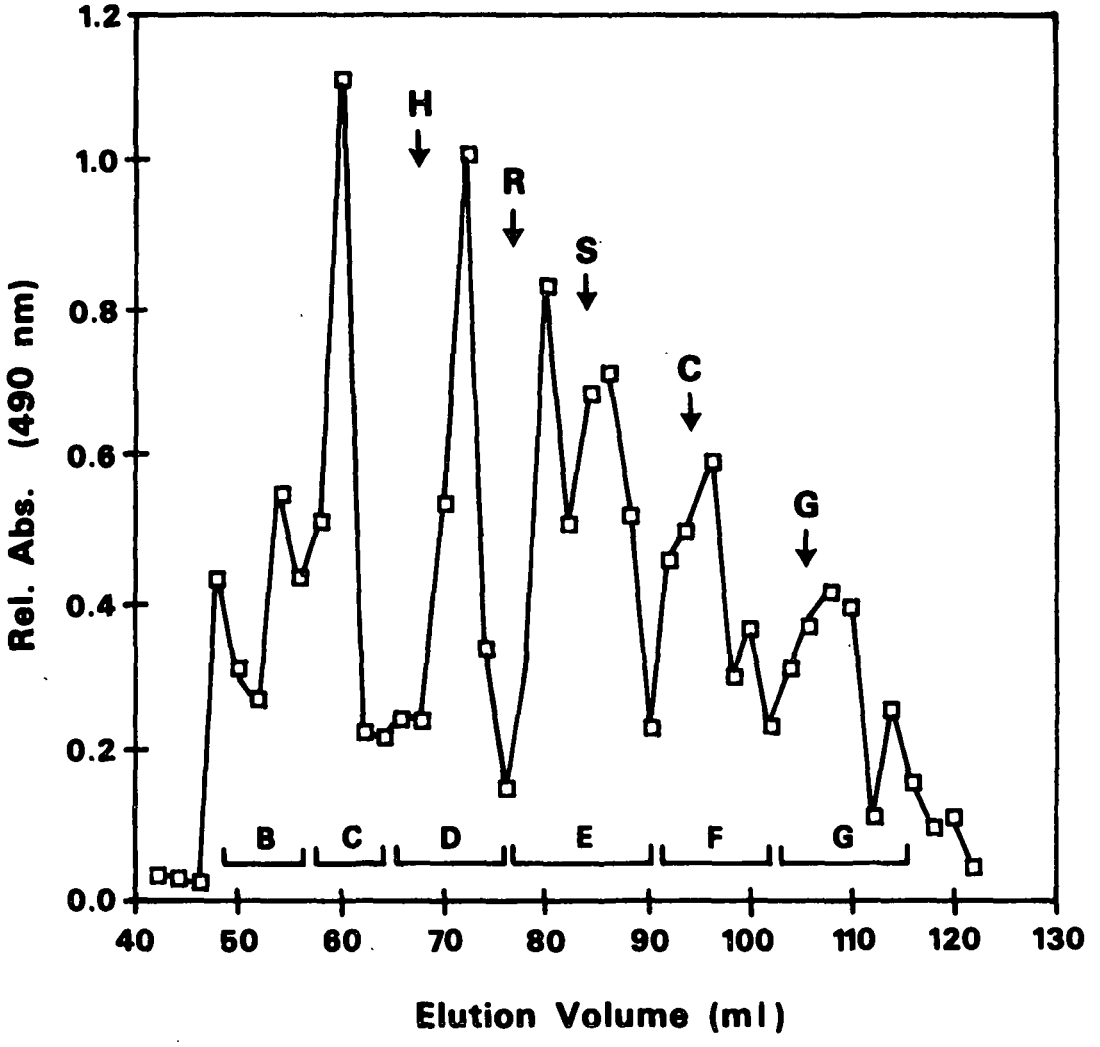

FIG. 5. Elution pattern of cellulase-treated DEXG-3 on Bio-Gel P-2. Points labeled H, R, S, C, G are the elution volumes of maltoheptose, raffinose, stachyose, cellobiose, and glucose, respectively.

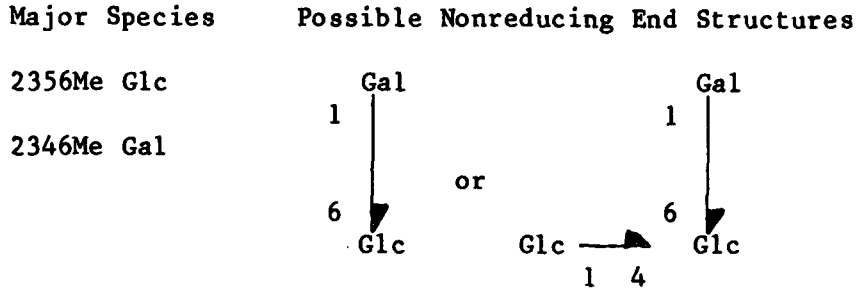

FIG. 6. Major components of fraction "C" and a possible structure for the nonreducing end.